

# Evaluation of four serological techniques to determine the seroprevalence of *Neospora caninum* in foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) on Prince Edward Island, Canada

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## Abstract

The objectives of this study were (1) to evaluate the performance and agreement of serological assays (ELISA, IFAT, *Neospora caninum* agglutination test and immunoblot) using reference sera and field sera from foxes and coyotes and (2) to estimate the *N. caninum* seroprevalence in foxes and coyotes on Prince Edward Island, Canada. With fox and coyote reference sera the test performance of the ELISA, IFAT and IB was excellent (100% sensitivity and specificity). NAT showed a low sensitivity (50%). Serum was collected from 201 coyotes and 271 foxes. The seroprevalence observed in the different assays ranged from 0.5 to 14.0% in coyotes and 1.1 to 34.8% in foxes. The seroprevalence, when taking more than one test positive as cut-off value was 3.3 and 1.1% for coyotes and foxes, respectively. From the *N. caninum*-positive group, all coyotes were older than 3 years. Agreement among assays (measured as prevalence-adjusted bias-adjusted kappa) using the field sera ranged from 0.17 to 0.97. Best agreement was observed between ELISA and IFAT, poor agreement was observed between NAT and the other assays. Positive agreement was moderate to poor among all assays utilized in this study. Although the seroprevalence observed was low, *N. caninum* antibodies are present in foxes and coyotes on Prince Edward Island (PEI) and their role in the *N. caninum* epidemiology needs further study. © 2007 Elsevier B.V. All rights reserved.

**Keywords:** Fox; Coyote; *Neospora caninum*; Serology; IFAT; ELISA; Immunoblot; *Neospora* agglutination test

## 1. Introduction

*Neospora caninum* is the most frequently diagnosed cause of abortion in cattle worldwide (Dubey, 1999).

Congenital infection appears to be the major mode of transmission in cattle but infection can also be caused by ingestion of oocysts shed by definitive hosts such as dogs and coyotes (Dijkstra et al., 2001, 2002; Gondim et al., 2004a).

Serological evidence for *N. caninum* infection in wild canids has been documented (Canon-Franco et al., 2004; Hamilton et al., 2005; Lindsay et al., 2001; Simpson

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et al., 1997). In North America, antibodies to *N. caninum* were detected in 5 (9.6%) of 52 coyotes (*Canis latrans*) in Texas (Lindsay et al., 1996) based on an IFAT, while 4 (15.4%) of 26 gray foxes (*Urocyon cinereoargenteus*) from a non-agricultural area in South Carolina had low titers to *N. caninum* (Lindsay et al., 2001), determined by a *N. caninum* agglutination test (NAT). Four out of 122 Alaskan wolves (*Canis lupus*) tested positive with the same NAT (Dubey and Thulliez, 2005). Antibodies against *N. caninum* were also detected based on an IFAT in 12 (10.6%) of 113 coyotes (*C. latrans*) from Utah, Colorado and Illinois (Gondim et al., 2004b). However, the accuracy of these prevalence estimates is unknown because test evaluations of assays for antibody detection of *N. caninum* in wild canids are not reported.

Assays to determine seroprevalence estimates of *N. caninum* in wild canids include IFAT, NAT, immunoblot (IB) and ELISA. IFAT and NAT are the most commonly used assays in seroprevalence studies in wild canids (Barber et al., 1997; Dubey and Thulliez, 2005; Gondim et al., 2004b). However, these studies typically collected only a limited number of samples. An ELISA is more rapid when dealing with large numbers of samples and is less dependent than IFAT on the experience of the diagnosticians performing the assay. NAT has the advantage that it is easy to read and can be applied to all host species within the same test run (Romand et al., 1998). IB is often used as an adjunct to other tests already in use, rather than as a tool for routine screening. However, IB is advantageous because it can identify immunodominant antigens (IDAs) that may still be evident when low titer sera are being tested (Atkinson et al., 2000; Schares et al., 2001a).

The Canadian province of Prince Edward Island (PEI) consists mainly of agricultural and forested land where coyotes and foxes co-occupy habitat throughout the island. In 2005, 24 (10%) dairy farms on PEI had an estimated within-herd *N. caninum*-seroprevalence of  $\geq 15\%$  (Wapenaar et al., in press). Coyotes and foxes are sighted close to farms and have access to carcasses and placentas. *Neospora*-like oocysts were observed in feces from two foxes (*Vulpes vulpes*) and two coyotes (*C. latrans*) on PEI (Wapenaar et al., 2006). Therefore, these two wild canid populations likely are reservoirs of *N. caninum*, but their seroprevalence levels have never been investigated.

The objectives of this study were (1) to evaluate the performance and agreement of serological assays (ELISA, IFAT, NAT, WB) using reference and field sera from foxes and coyotes and (2) to estimate the *N. caninum*-seroprevalence in foxes and coyotes on Prince Edward Island.

## 2. Materials and methods

### 2.1. Reference samples

Reference sera from 10 *N. caninum*-negative coyotes (*C. latrans*) were received from Dr. William Pitt (Hilo, HI, USA) and Dr. Nohra Mateus-Pinilla (Urbana, IL, USA). Sera from six *N. caninum*-positive coyotes were provided by Dr. Pita Gondim (Salvador, Brazil), consisting of four experimentally infected coyotes and two field sera. The field sera were determined *N. caninum*-positive by IFAT in a previous study (Gondim et al., 2004b).

Ten ranched silver foxes (*V. vulpes*) from the Canadian Centre for Fur Animal Research (Nova Scotia Agricultural College, Truro, Canada), were used to obtain *N. caninum*-negative and *N. caninum*-positive reference sera. After primary blood collection, all 10 foxes were vaccinated with a commercially available bovine *N. caninum*-vaccine (Neoguard<sup>®</sup>, Intervet, Whitby, ON, Canada). Five animals were given a booster vaccination 3 weeks after initial vaccination. Blood was collected again from all 10 animals 6 weeks after initial vaccination.

### 2.2. Field samples

Coyote and fox carcasses were obtained via 32 registered hunters and trappers from across PEI, during their normal hunting and trapping activities from 19 October, 2004 until 24 March, 2005. No foxes or coyotes were killed deliberately for this study.

Approximately 10 ml of blood were collected from the heart or femoral artery of each carcass into a vacutainer tube. All carcasses were sampled as soon as possible after death, varying from a few hours to 5 days. Most carcasses were sampled within 24 h after death at the location where they were skinned and stored. Within 24 h of collection, blood samples were centrifuged at  $1000 \times g$  for 10 min. Based on visual assessment of the severity of hemolysis, the serum quality was recorded as 'good' or 'bad'. The serum samples were stored at  $-20^\circ\text{C}$  until all samples were obtained.

The canine tooth of the lower jaw was collected and sex was recorded for each coyote and fox carcass. To differentiate between a juvenile ( $<12$  months) and an adult animal, radiographs were taken of individual canine teeth to assess width of the pulp cavity. The age of adult animals was determined by counts of the annual growth zones in the canine tooth cementum (Johnston Biotech, Sarnia, Ontario, Canada) (Grue and Jensen, 1976; Johnston et al., 1999).

### 2.3. Laboratory analyses

The coyote and fox reference and field sera were tested with an indirect ELISA (Biovet Inc., St. Hyacinthe, Canada), commercially available for use in bovine sera, according to the manufacturer's recommendations (Paré et al., 1995), but with a few modifications involving the conjugate and positive and negative control samples. As conjugate, rabbit anti-dog IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) was used (dilution 1:5000) instead of the bovine conjugate provided with the kit. Pooled serum samples from each of the coyote and fox reference sera were used as positive and negative control for each species. Serum samples were tested in duplicate (dilution 1:200) and the optical density (OD) was measured in a microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 405 nm. The results were reported as a sample-to-positive ratio (S/P-ratio; (average sample OD – blank OD)/(positive OD – blank OD)). Cut-off values were selected using positive and negative reference sera as described below.

An IFAT (1:25 dilution) was performed on the same coyote and fox field sera and the fox reference sera at the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD, USA, as described by Dubey et al. (1996). The coyote reference sera could only be used in the ELISA due to the limited volume of serum available. The intensity of fluorescent antibody bound to the periphery of the tachyzoite was recorded on a categorical scale from negative to strong fluorescence (0 to 4+) by an experienced reader. Fluorescence of the periphery of the tachyzoite recorded as 0 was considered as *N. caninum*-negative result, fluorescence of the periphery of the tachyzoite recorded as  $\geq 1+$  was considered *N. caninum*-positive.

A NAT (1:25 and 1:100 dilution) was performed on the coyote and fox field sera and the fox reference sera at APDL, as described by Romand et al. (1998).

An IB (1:100 dilution) was performed on the coyote and fox field sera and the fox reference sera at FLI (Wusterhausen, Germany), as described (Schares et al., 1998, 1999, 2001a), but with a few modifications. A 1:1000 dilution of the conjugate (Anti-dog IgG(H + L) peroxidase conjugate, Dianova, Germany) was used. The IB result was regarded as positive when at least two of five immunodominant antigens (IDAs, with molecular weights of 19, 29, 30, 33 and 37 kDa) were recognised (Schares et al., 2001a). If only one band was recognized, an inconclusive test result was recorded.

As a confirmatory test, 94 field sera (inconclusive results between ELISA, IFAT, NAT or IB) were analysed in an additional immunoblot (IB38), using the p38 surface antigen of NC-1 tachyzoites, purified as described by Schares et al. (2000). P38 (0.4  $\mu$ g) was treated for 10 min at 96 °C with sample buffer (2% [w/v] sodium dodecyl sulfate (SDS), 10% [v/v] glycerol, 62 mM Tris-HCl, pH 6.8) and electrophoresed in a 9.5 cm wide SDS-polyacrylamide gel (12.5% [w/v]). Separated antigen was electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore) in a semi-dry transfer system (Pharmacia Biotech, Freiburg, Germany), using a current of 1.5 mA/cm<sup>2</sup> gel for 90 min. After blocking with PBS-T-Gelatine (PBS, 0.05% [v/v] Tween 20, 1% [w/v] gelatine) the membrane was cut into 50 stripes and stored frozen at –20 °C until used to analyse the serum samples.

### 2.4. Statistical analyses

Likelihood ratio and two-graph receiver operating characteristic (TG-ROC) analyses were performed to determine the optimal cut-off value and sensitivity (Se) and specificity (Sp) of the ELISA, using coyote and fox reference sera.

Prevalence-adjusted bias-adjusted kappa (PABAK) was calculated to determine agreement (beyond that due to chance) between the different assays, using field sera (Byrt et al., 1993). Positive and negative agreements were calculated to assess in more detail where disagreements among test results occurred (Cicchetti and Feinstein, 1990). Stata Version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses (likelihood ratio and TG ROC) of the data.

## 3. Results

### 3.1. Test validation using reference samples

Good discrimination between the *N. caninum*-positive and *N. caninum*-negative reference samples was observed for coyotes and for foxes (Fig. 1). The S/P ratios of *N. caninum*-positive reference samples from the coyotes ( $n = 6$ ) ranged from 0.48 to 1.41, with a mean value of 1.00 (median = 1.08). The S/P ratios of *N. caninum*-negative reference samples from the coyotes ( $n = 10$ ) ranged from 0.02 to 0.36, with a mean value of 0.11 (median = 0.07). The S/P ratios of *N. caninum*-positive reference samples from the foxes ( $n = 10$ ) ranged from 0.20 to 1.03, with a mean value of 0.73 (median = 0.82). The S/P ratios of *N. caninum*-negative

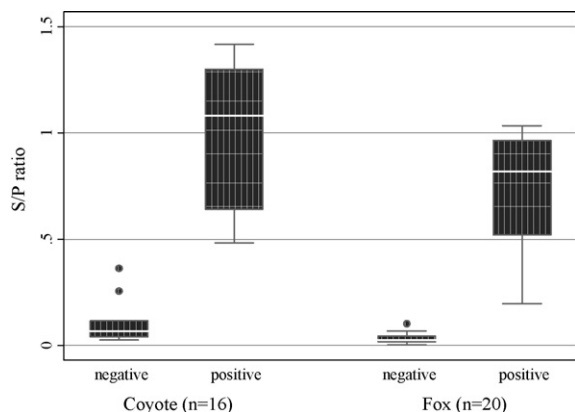


Fig. 1. Box and whiskers plot of *Neospora caninum* reference sera from coyote sera ( $n = 16$ ) and fox sera ( $n = 20$ ) analysed by indirect ELISA. The horizontal white line in the box indicates the median, the box indicates the 25th and 75th percentile, and the whiskers indicates the upper and lower adjacent values. Dots represent outside values (Tukey, 1977).

reference samples from the foxes ( $n = 10$ ) ranged from 0.00 to 0.10, with a mean value of 0.03 (median = 0.02).

For coyotes, the likelihood ratio analysis determined the optimal cut-off value to be  $\geq 0.40$ , producing a Se of 1.00 (95% CI: 0.54–1.00) and Sp of 1.00 (95% CI: 0.69–1.00). A cut-off value  $\geq 0.19$  was determined to be optimal when analysing sera of foxes, producing a Se of 1.00 (95% CI: 0.69–1.00) and Sp of 1.00 (95% CI: 0.69–1.00).

The IFAT and IB also had a Se of 1.00 (95% CI: 0.69–1.00) and Sp of 1.00 (95% CI: 0.69–1.00). The IB38 showed 1 inconclusive result for a positive reference sample, resulting in a Se of 0.90 (95% CI: 0.56–1.00) and Sp of 1.00 (95% CI: 0.69–1.00). The

NAT (dilution 1:25) had a Se of 0.50 (95% CI: 0.19–0.82) and Sp of 1.00 (95% CI: 0.69–1.00).

### 3.2. Field samples

#### 3.2.1. Descriptive information

Serum was collected from 472 wild canids comprised of 201 coyotes and 271 foxes. Due to insufficient volume, not all sera were tested in all assays, as described in Table 2. Sex was recorded for 426 animals, and was roughly equally divided among both species; 92 female and 88 male coyotes, and 115 female and 131 male foxes. The age determination showed that 48% ( $n = 89$ ) and 58% ( $n = 158$ ) of the coyotes and foxes, respectively, were less than 12 months of age. Twenty-six coyotes (14%) and 18 foxes (7%) were over 4.5 years of age. Serum quality of 55% of sera collected was recorded as ‘bad’ due to severe hemolysis. For the foxes, 133 (49%) sera were ‘bad’, whereas 127 (63%) sera of coyotes were ‘bad’.

#### 3.2.2. Seroprevalence in foxes and coyotes

The seroprevalence observed in the different assays ranged from 0.5 to 14.8% in coyotes and 1.1 to 34.8% in foxes (Table 1). The highest prevalence estimates were found with NAT (14.8 and 34.8% for coyotes and foxes, respectively). The lowest prevalence estimates (0.5 and 1.1% for coyotes and foxes, respectively) were observed with IFAT. Only six coyotes and five foxes were positive in more than one assay (Table 2). For this analysis, animals that tested positive only in IB and IB38 were considered negative, since the IB38 was used as a confirmatory test on a small sample set and had a similar technique as the IB. The NAT (1:100) was not seen as separate test, because the NAT (1:25) was included and

Table 1

Seroprevalence (with 95% confidence interval) of *Neospora caninum* in coyotes and foxes on Prince Edward Island, Canada, evaluated in five different assays

Test <sup>a</sup>	Coyotes		Foxes		Total
	No. tested	% pos (95% CI)	No. tested	% pos (95% CI)	% pos (95% CI)
ELISA	201	2.5 (0.8–5.7)	271	2.6 (1.0–5.2)	2.5 (1.3–4.4)
IFAT	201	1.5 (0.3–4.3)	271	1.1 (0.2–3.2)	1.3 (0.5–2.7)
NAT (1:25)	183	14.8 (9.9–20.7)	270	34.8 (29.1–40.8)	26.7 (22.7–31.0)
NAT (1:100)	183	0.5 (0.00–3.0)	270	5.6 (3.1–9.0)	3.5 (2.0–5.7)
IB	198	6.0 (3.2–10.3)	264	1.5 (0.4–3.8)	3.5 (2.0–5.6)
IB38 <sup>b</sup>	33	21.2 (9.0–38.9)	61	9.8 (3.7–20.2)	13.8 (7.5–22.5)
>1 test <sup>c</sup>	181	3.3 (1.2–7.1)	263	1.1 (0.2–3.3)	2.0 (0.9–3.8)

<sup>a</sup> NAT, *N. caninum* agglutination test; IB, immunoblot.

<sup>b</sup> Seroprevalence estimate is not representative for the whole population because only 94 sera were tested in IB38 that were inconclusive based on other assay results.

<sup>c</sup> Sample was considered positive when more than one test out of four (ELISA, IFAT, NAT (1:25), IB) was found positive. IB38 was not included because a specific subset of samples was used in this assay.

Table 2

Descriptive data of 11 *Neospora caninum*-seropositive coyotes and foxes, which tested positive in more than one assay. A sample was considered positive when more than one test out of five (ELISA, IFAT, NAT (1:25), IB or IB38) was found positive. A sample was considered negative if it tested positive only for IB and IB38.

Species	Age (year)	Sex	Serum quality	ELISA <sup>a</sup>	IFAT	NAT 1:25	IB <sup>b</sup>	IB38	No. tests positive
Coyotes									
1	3.5	♀	Bad	<b>0.46</b>	+	–	+(29,30,33,37)	+	4
2	4.5	♀	Bad	<b>0.71</b>	–	–	+(29,30,33,37)	+	3
3	4.5	♂	Bad	<b>0.61</b>	–	–	+(29,30,33,37)	+	3
4	5.5	♀	Bad	0.18	–	+	+(29,30,37)	+	3
5	6.5	♂	Bad	<b>0.91</b>	+	–	+(29,29,30,33,37)	+	4
6	13.5	♀	Good	<b>1.61</b>	+	–	+(29,30,33,37)	+	4
Foxes									
1	0.5	♀	Good	0.12	–	+	–	+	2
2	0.5	♂	Good	0.18	–	+	–	+	2
3	0.5	♂	Good	<b>0.22</b>	–	+	–	–	2
4	1.5	♀	Good	<b>0.56</b>	+	+	–	–	3
5	5.5	♀	Good	<b>0.27</b>	+	+	–(37)	–	3

<sup>a</sup> Bold printed numbers are positive ELISA results.

<sup>b</sup> Numbers in brackets indicate which positive bands were observed.

these assays were only differing in serum dilution. From this group of *N. caninum*-positive animals, all positive coyotes were older than 3 years of age. Conversely, three out of five *N. caninum*-positive foxes were less than 1 year of age. Gender and serum quality were equally distributed among the group.

### 3.2.3. Test agreement

When analysing agreement among tests using the field sera, PABAKs among all assays ranged between 0.17 and 0.97 (Table 3). Positive and negative agreement ranged between 0 and 0.56, and 0.72 and 0.99, respectively. There was poor agreement between NAT and the other assays, greatest agreement was observed between ELISA and IFAT (PABAK 0.97,  $P_{\text{pos}}$  0.56,  $P_{\text{neg}}$  0.99). Agreement between the ELISA and other assays was calculated for foxes and coyotes separately in Table 4, because a different cut-off value was used for each species. Positive agreement between the ELISA, IFAT and IB was higher in coyotes than in foxes.

## 4. Discussion

Most assays performed excellent with the reference coyote and fox sera, with exception of the NAT. No explanation is known for the poor sensitivity of the NAT observed in the experimentally generated reference sera. Because most reference *N. caninum*-positive animals were experimentally infected (coyotes) or vaccinated (foxes), the high Se and Sp for the other tests are likely to be overestimated. Antibody response determined by a serological assay can be significantly stronger in experimentally infected animals compared

Table 3

Prevalence-adjusted, bias-adjusted kappa (PABAK<sup>a</sup>), positive ( $P_{\text{pos}}$ <sup>a</sup>) and negative ( $P_{\text{neg}}$ <sup>a</sup>) test agreement between ELISA, IFAT, NAT (1:25 and 1:100 dilution), IB and IB38 to detect *N. caninum* antibodies, performed on field sera from coyotes and foxes

Test <sup>b</sup>	ELISA	IFAT	NAT (1:25)	NAT (1:100)	IB
IFAT					
PABAK	<b>0.97<sup>c</sup></b>				
$P_{\text{pos}}$	0.56	–			
$P_{\text{neg}}$	<b>0.99</b>				
NAT (1:25)					
PABAK	0.44	0.45			
$P_{\text{pos}}$	0.05	0	–		
$P_{\text{neg}}$	<b>0.84</b>	<b>0.98</b>			
NAT (1:100)					
PABAK	<b>0.88</b>	<b>0.90</b>	0.53		
$P_{\text{pos}}$	0	0.03	0	–	
$P_{\text{neg}}$	<b>0.97</b>	<b>0.84</b>	<b>0.84</b>		
IB					
PABAK	<b>0.92</b>	<b>0.93</b>	0.41		<b>0.87</b>
$P_{\text{pos}}$	0.33	0.27	0.01		0
$P_{\text{neg}}$	<b>0.98</b>	<b>0.98</b>	<b>0.83</b>		<b>0.97</b>
IB38 <sup>d</sup>					
PABAK	0.74	0.70	0.17	0.46	0.68
$P_{\text{pos}}$	0.50	0.22	0.18	0.14	0.44
$P_{\text{neg}}$	<b>0.93</b>	<b>0.92</b>	0.72	<b>0.84</b>	<b>0.91</b>

<sup>a</sup> PABAK,  $P_{\text{pos}}$ ,  $P_{\text{neg}}$  interpretation: <0.2 slight agreement, 0.2–0.4 = fair agreement, 0.4–0.6 = moderate agreement, 0.6–0.8 = substantial agreement and >0.8 = almost perfect agreement (Dohoo et al., 2003).

<sup>b</sup> NAT, *N. caninum* agglutination test; IB, immunoblot.

<sup>c</sup> Bold printed numbers indicate almost perfect agreement.

<sup>d</sup> Not comparable because only 94 sera were tested in IB38 that were inconclusive based on other assay results.



Table 4

Prevalence-adjusted, bias-adjusted kappa (PABAK<sup>a</sup>), positive (P<sub>pos</sub><sup>a</sup>) and negative (P<sub>neg</sub><sup>a</sup>) test agreement specified per species between ELISA and other assays (IFAT, NAT (1:25 and 1:100 dilution), IB and IB38) to detect *N. caninum* antibodies, performed on field sera from coyotes and foxes

ELISA <sup>b</sup>	Fox	Coyote
IFAT		
PABAK	0.96	0.98
P <sub>pos</sub>	0.40	0.75
P <sub>neg</sub>	0.99	0.99
NAT (1:25)		
PABAK	0.30	0.65
P <sub>pos</sub>	0.06	0
P <sub>neg</sub>	0.78	0.90
NAT (1:100)		
PABAK	0.84	0.93
P <sub>pos</sub>	0	0
P <sub>neg</sub>	0.96	0.98
IB		
PABAK	0.92	0.93
P <sub>pos</sub>	0	0.59
P <sub>neg</sub>	0.98	0.98
IB38 <sup>c</sup>		
PABAK	0.70	0.82
P <sub>pos</sub>	0.31	0.73
P <sub>neg</sub>	0.92	0.95

<sup>a</sup> PABAK, P<sub>pos</sub>, P<sub>neg</sub> interpretation: <0.2 slight agreement, 0.2–0.4 = fair agreement, 0.4–0.6 = moderate agreement, 0.6–0.8 = substantial agreement and >0.8 = almost perfect agreement (Dohoo et al., 2003).

<sup>b</sup> NAT, *N. caninum* agglutination test; IB, immunoblot.

<sup>c</sup> Not comparable because only 94 sera were tested in IB38 that were inconclusive based on other assay results.

to those infected naturally (Matsushita et al., 1987). By collecting serum from foxes 6 weeks after vaccination, high antibody levels can be anticipated, especially in the animals that received a second booster vaccination. In the field, animals may have been infected with *N. caninum* several years before blood was collected and subsequently their titer may be low at the time of measurement. In addition, fluctuation of *N. caninum*-antibody titers, as demonstrated in cattle (Conrad et al., 1993), may also occur in wild canids.

Although the ELISA used sonicated tachyzoites from bovine origin as antigen, the test performance was excellent with sera from foxes and coyotes in the same serum dilution. For cattle, cut-off S/P-ratios of 0.45 and 0.60 are recommended. Our optimal cut-off S/P-ratios of 0.19 and 0.40 are lower than for cattle, suggesting a weaker antibody response. However, this weaker response may be due to the lower affinity of the anti-canine antibody used as conjugate to the coyote and fox

antibodies. The fact that coyotes had higher S/P ratios than foxes supports this, because the coyote (*C. latrans*) is genetically more closely related to the dog (*Canis familiaris*) than the fox (*V. vulpes*).

Agreement among the different assays ranged from poor to almost perfect. Disagreement was mainly due to none or very poor agreement among the positive sera (P<sub>pos</sub>, Table 1). However, due to the low number of positive samples, positive agreement estimates are obtained from only a small number of positive samples.

It was interesting to note that all but four *N. caninum*-positive animals were 3.5 years of age and older. In fact, all of the *N. caninum*-positive coyotes were 3.5 years or older. This was in contrast with the general age structure in the collected population where approximately 50% of the animals were under 1 year of age. The age distribution of the collected animals may not reflect the true age distribution of the wild population; young animals are more likely to be successfully trapped or hunted than older animals. Therefore, the calculated prevalences may be underestimated because there was the trend of increased seropositivity with age. The *N. caninum*-seroprevalence in juveniles was 1.2% (95% CI: 0.3–3.5), and the seroprevalence in animals older than 1 year was 3.8% (95% CI: 1.6–7.3). Based on these age-stratified seroprevalences, horizontal transmission is likely to be the primary source of infection in these wild canid populations on PEI. For both foxes and coyotes there were more females than males *N. caninum*-seropositive (Table 2). However, this difference was not significant, and more seropositive field samples are needed to assess a possible sex-related distribution.

Although the observed seroprevalence was low in most assays, the present study documents the occurrence of antibodies to *N. caninum* in wild canids on PEI. Low seroprevalence (0–2%) has previously been reported among red foxes in the UK (Hamilton et al., 2005), Ireland (Wolfe et al., 2001), Sweden (Jakubek et al., 2001) and Austria (Wanha et al., 2005). The serologic evidence of *N. caninum* infection in the coyotes and foxes, poses questions about the relative importance of transmission of *N. caninum* between wildlife and livestock. The role of other wildlife (i.e. birds, rodents) in the *Neospora*-lifecycle needs to be investigated. Low seroprevalence does not necessarily imply a low risk of shedding oocysts; studies have shown that the majority of dogs that shed oocysts showed no seroconversion either in IFAT or IB (Schares et al., 2001b). In addition, autolysis of the sample (collected post-mortem) may have caused degradation of immunoglobulins, and prevalence could therefore have been underestimated.

Seroprevalences observed with the four different techniques were not very different except for the NAT. Cross-reactivity with antibodies against *Sarcocystis cruzi* has been reported for the NAT (Romand et al., 1998); this may have influenced the high seroprevalence estimates observed in our field samples. Although the IFAT had an excellent sensitivity with the control sera, the seroprevalence was the lowest, in comparison with the other assays (Table 2). A moderate Se of the IFAT using a low cut-off has been observed in other studies, using fetal fluids (Söndgen et al., 2001; Wouda et al., 1997). Although the seroprevalence with IB, ELISA and IFAT was in the same range, the poor test agreement shows that they do not classify the same animals as seropositive. However, agreement between positive coyotes was considerably higher than between positive foxes when comparing the ELISA with most other assays ( $P_{\text{pos}}$ , Table 4). It is important to identify the cause of these differences, if one desires to use these assays to obtain results on individual animals. Cross-reactivity in assays could be a concern, as sera from cattle infected with *Toxoplasma gondii* and *Sarcocystis* spp. had multiple cross-reacting antibodies recognizing *Neospora* antigen including antigens of 31 and 37 kDa when studied by Baszler et al. (1996). Dubey et al. (1996) also reported cross-reactivity of sera from *Sarcocystis*-infected cattle by indirect ELISA. *Sarcocystis* spp. are common in cattle in Canada and it would therefore be expected to find antibodies against *Sarcocystis* spp. in wild canids. The IFAT utilizes a surface antigen and the degree of fluorescence needs to be assessed by an experienced reader, which may explain the different results obtained. The sonicated tachyzoite utilized as antigen in the ELISA may be more sensitive than the whole tachyzoites used in the IFAT because there is exposure of both internal and surface antigens. However, due to possibly identical antigens presented, a decreased Sp is expected for the ELISA because there is a greater possibility for cross-reactivity with other protozoa, such as *Hammondia* spp. It has been suggested that in addition to IFAT and ELISA techniques, other tests should be performed, such as immunoblotting, because of a potential confounding factor *Hammondia heydorni*, the closest phylogenetically related protozoan parasite to *N. caninum* (Gondim, 2006; Staubli et al., 2006). Furthermore, in a recent study, IB was confirmed to be superior in its Se and Sp when compared to ELISA (Staubli et al., 2006). This finding could not be confirmed with our field samples and the overall prevalence estimate of 3.5% found in this study was the same for IB and NAT (1:100) and was only slightly

higher than for ELISA (2.5%) and IFAT (1.3%). A different study has suggested that the 37 kDa antigen detected by IB may be identifying latent infections (Bjerkas et al., 1994). Hence, it was interesting to note that from the 20 animals that had a positive band at the 37 kDa antigen, 17 were determined to be animals older than 1 year. Because the true nature of the sample is not known, we cannot make assumptions about Se and Sp of each assay when using field samples, since IFAT, ELISA and IB performed excellent with the reference sera. More research is needed to explain the differences in test results observed in this study. Also, our results indicate that one has to be careful interpreting test data evaluated with only one assay, especially when making assumptions about individual animals.

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